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A COMPARISON OF TWO METHODS FOR DISPERSING WHOLE HUMAN SALIVA FOR MICROFLORAL ASSAY

by

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Bureau of Medicine and Surgery, Navy Department
Research Work Unit M4311.02-1002.01

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SUMMARY PAGE

THE PROBLEM

To compare two methods for dispersing human saliva for microfloral assay, and to determine correlations between bacterial counts in human saliva following specimen processing. The methods to be compared were that of Jay Shaker*, and the Vortex Genie** method.

FINDINGS

Bacterial counts derived from several different media revealed that in general, no significant differences existed between the two different dispersion techniques employed. Strong correlations were found between counts obtained by the two methods.

APPLICATIONS

Considering the factors of portability, compactness, and decreased processing time, it is felt that the Vortex dispersion technique is to be preferred to the Jay method for purposes of general laboratory and field utilization.

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* Eberbach Corporation, Ann Arbor, Michigan
** Scientific Industries, Inc., Springfield, Massachusetts

ADMINISTRATIVE INFORMATION

This investigation was conducted as part of Bureau of Medicine and Surgery Research Work Unit M4311.02-1002. The present report is Number 1 on this work unit. It was submitted for review on 28 Feb 1975, approved for publication on 14 April 1975, and designated as NavSubMedRschLab Report No. 807.

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ABSTRACT

Certain bacteria commonly present in the normal oral flora, exhibit an agglutination phenomenon or "clumping" of cells. This condition seen in pure cultures of some bacteria is compounded by the interaction of mucin, a substance inherently present in saliva, making accurate representations of salivary bacterial densities difficult to obtain.

Various methods of counteracting this agglutination effect have been investigated, ranging from the treating of saliva with dilute sodium hydroxide or 1% horse serum to a variety of mechanical dispersion techniques.

Although none of the techniques studied have proven completely satisfactory, the method most often employed in quantitative experiments has traditionally been that of mechanical shaking described by Jay in 1927. A newer and more efficient instrument, the Vortex Genie, is now commonly used in laboratories to disperse and suspend bacterial cultures. Most workers in the field of salivary bacteriology are reluctant to adopt this instrument since comparisons with the traditional technique are not available. Because the Vortex Genie is more economical in time, space and money, this study sought to determine correlations between the two methods as a basis for using the Vortex Genie in future salivary bacterial research.

Bacterial assays of whole saliva specimens collected from 40 subjects were utilized as indicators for establishing correlations. The two dispersion techniques were used and specimens were plated on five different selective media as well as one enriched type medium. The strong correlation between paired plate counts for the Jay and Vortex-treated saliva samples demonstrated a practical and useful correspondence between the two methods.

A COMPARISON OF TWO METHODS FOR DISPERSING WHOLE HUMAN SALIVA FOR MICROFLORAL ASSAY

INTRODUCTION

The difficulties involved in obtaining quantitative representations of salivary bacteria have been well-documented.[1,2,3] Most commonly studied oral bacteria exhibit an agglutination or "clumping" phenomenon. This characteristic has been noted in pure cultures of these bacteria. The problem of obtaining salivary bacterial counts is compounded by the common growth of several of these strains in a single saliva sample, together with the interaction of salivary mucin. Accurate counts and reliable duplication of results are often difficult to obtain.

Various methods of counteracting this agglutination effect have been investigated. Crowley and Rickert[1] devised a compressed air atomizer to break up clumps and sought to dissolve mucin with a dilute solution of sodium hydroxide. This method, however, produced an overall 13% difference between duplicate plate counts made from each of 34 samples.

Bibby and Maurer[2] observed bacterial "clumps" microscopically and used a hemacytometer to obtain direct counts. Whereas culturing of raw saliva produced colony counts averaging only 16.8% of the direct microscopic count, the addition of 1% horse serum to reduce clumping resulted in a sixfold increase in counts. Concurrent use of mechanical dispersion was not reported.

More recently, Williams and Eickenberg[3] demonstrated that consistently higher counts of oral bacteria could be obtained by using sonic vibration (9000 cycles per second with a power output of 50 watts) as the vehicle of dispersion. However, high frequency vibrations have also been used to disintegrate bacterial cells for enzyme extraction.[4] Moreover, Beckwith and Weaver[5] have investigated possible lethal effects of ultrasound on certain strains of yeast and bacteria. Because of the implications of these studies, it is difficult at this time to assess the value of high frequency bacterial dispersion in saliva. The possibility of destroying oral bacteria indicates caution in the use of ultrasound techniques in assay methods requiring growth of organisms. One must also consider the possibility of metabolic changes in response to ultrasound. This aspect has not been fully investigated.

The most consistently used technique for the dispersion of whole saliva continues to be the mechanical Jay Shaker. The disadvantages of using the Jay Shaker technique include:

1. Specimen dilutions must be maintained in suspension with a Kahn Platform Shaker* after dispersion on a Jay Shaker.

2. The Kahn Platform Shaker is an additional and bulky piece of equipment.

**Eberbach Corporation, Ann Arbor, Michigan*

3. Relatively large volumes of diluent must be used.

4. Specimen processing is relatively time consuming.

The Vortex Genie has found common acceptance in many bacteriological laboratories.[7] Employing a whirlpool-type motion, it is routinely used for the resuspension of bacterial broth culture and in preparing dilutions. The use of this compact device provides the investigator a speedy and economical technical tool.

This study seeks to determine correlations between human oral salivary bacterial counts following specimen processing by use of the Jay Shaker and the Vortex Genie.

MATERIALS AND METHODS

1. Collection of Saliva

a. A paraffin stick (melting point 50°-52° C) was allowed to soften in the mouth and then masticated to produce salivary stimulation. The stimulated saliva was then collected in a sterile 4 dram vial. Approximately 15 ml of saliva was collected from each of 40 male subjects in the submarine service, ages 23-41, who were undergoing comprehensive physical examination. All specimens were collected in the morning immediately upon rising and before taking anything by mouth.

2. Treatment of Saliva

Two methods of treating each saliva specimen were employed. Both techniques were mechanical in nature and differed principally in intensity and duration of action.

a. Mechanical Shaking: A Jay Shaker (Eberbach Corporation, Ann Arbor, Michigan) was used which was designed to deliver 85 cycles per second. Five ml of whole saliva was delivered to a sterile 4 dram vial containing 4 glass beads (5 mm in diameter) and shaken for a period of 5 minutes (Jay-treated).

b. Vortexing: A Vortex Genie (Scientific Industries, Inc., Springfield, Massachusetts) was used which provided a continuous whirlpool motion of the sample. Five ml of whole saliva was delivered to a like sterile 4 dram vial containing 4 glass beads and vortexed for a period of 30 seconds (Vortex-treated).

3. Dilution Methods

Both the Jay-treated saliva and the Vortex-treated saliva were diluted serially in 0.1% Yeast Extract Broth (YEB) (DIFCO, Detroit, Michigan).

a. Dilution of Jay-treated saliva: An aliquot of 1.0 ml Jay-treated saliva was used. Since 5 minutes of shaking time were required for preparation of the dilutions (10^{-1}-10^{-6}), a Kahn Shaker (Eberbach Corporation, Ann Arbor, Michigan) with a platform designed to accommodate 99 ml dilution blanks was also used. Dilutions were then prepared by pipetting 1.0 ml of the Jay-treated saliva to 9 ml YEB and shaking for 5 minutes (10^{-1}). Concurrently, 1.0 ml of Jay-treated saliva was transferred to 99 ml of YEB and shaken for 5 minutes on the Kahn Shaker (10^{-2}). Subsequent dilutions were prepared by transferring 1.0 ml of the 10^{-1} dilution to another 99

ml blank preparing the 10^{-3} dilution, 1 ml of the 10^{-2} dilution to make 10^{-4} dilution, etc. until the series of 10^{-1} through 10^{-6} was completed. Each dilution was shaken on the Kahn Shaker for a total of 5 minutes before aliquots were transferred to the appropriate dilution blank and agar plate.

b. Dilution of Vortex-treated saliva: A 1.0 ml aliquot of Vortex-treated saliva was serially diluted in 9.0 ml blanks of 0.1% YEB. Each dilution was mixed by vortexing for 30 seconds prior to transfer to the next dilution tube in the series (10^{-1} -10^{-6}). The time required using this procedure was only three minutes per sample. Each dilution was then resuspended on the Vortex for 5 seconds immediately prior to plating.

4. Media, and Colony Counts

Commercially prepared media (DIFCO, Detroit, Michigan) were used throughout this study. Total Colony Counts (TOT) were obtained using Brain Heart Infusion Agar (BHI). Streptococcus salivarius counts (SAL) were determined on Mitis-Salivarius Agar (MSA). Additionally, Streptococcal types other than Streptococcus salivarius (OST) were also estimated from MSA. Mannitol Salt Agar (MAN) was used to presumptively quantitate Staphylocci-Micrococci (STM). Yeast counts (YST) were determined by the use of Sabouraud Dextrose Agar (SAB) and Lactobacilli (LAC) were quantitated using Rogosa Agar (ROG). Presumptive identification was based primarily on growth characteristics on selective/differential media.

5. Plate Counting Technique

Treated saliva samples were diluted 10^{-1} through 10^{-6} as described above. Aliquots of 0.1 ml of the appropriate dilutions were spread over the surface of the media using metal spreaders. Duplicate plates were prepared in each case. Experience in this laboratory has shown that not all of the dilutions need be plated for each medium used. Accordingly, dilutions were selected which would bracket the 30-300 colony counting range.

Streptococcus salivarius counts were determined after 24 hours from the 10^{-6} and 10^{-7} dilutions. These plates were then reincubated for a total of 5 days at which time OST were determined. For the Total Colony Count, 10^{-5}, 10^{-6} and 10^{-7} dilutions were plated and incubated for 48 hours. Yeast counts were determined after 48 hours from the 10^{-2} and 10^{-3} dilutions. Dilutions of 10^{-2} and 10^{-3} were also used for the Staphylococci-Micrococci which were counted at 72 hours. Since Lactobacilli are often present in widely varying numbers depending on the subject sampled, dilutions of 10^{-2} through 10^{-5} were used. Lactobacillus counts were determined after 5 days incubation. All plates were incubated at 37°C.

6. Statistical Methods

Duplicate plate counts obtained on each subject were averaged and the resulting numbers analyzed by two methods:

(1) Summations were made for the entire study group and mean total counts determined for each bacterium or type assayed. The mean values representing

total counts obtained by each dispersion method were then subjected to statistical analysis for significance of their differences by calculating t-values for correlated samples.[8]

(2) Correlation coefficients (r) relating the two dispersion methods were determined from corresponding counts of each bacterium or type.[8]

RESULTS

Mean Colony count values with corresponding correlation coefficients for paired determinations are shown in Table I.

In every instance mean bacterial counts per milliliter of Vortex-treated saliva were greater than the mean bacterial counts per milliliter of Jay-treated saliva. In only one instance was this difference statistically significant. The mean OST count from Vortex-treated saliva was greater than the mean count of OST from Jay-treated saliva at the 0.05 level of confidence. This data is graphically illustrated in Figure 1.

Table I. Mean colony count values with corresponding correlation coefficients on various media after dispersion of whole saliva by Vortex and Jay Shaker techniques.

Medium and Type Counted	Dispersion Method				Correlation Coefficient
	Vortex		Jay		
	Mean	SEM[a]	Mean	SEM	
Brain Heart Infusion					
Total Colony Count (TOT)	287.5	± 50.3	238.5	± 28.8	0.82
Mitis-Salivarius					
Streptococcus salivarius (SAL)	67.9	± 9.3	58.9	± 7.2	0.62
Other Streptococci (OST)	142.9	± 20.3	107.3[b]	± 12.4	0.44
Rogosa SL					
Lactobacilli (LAC)	107.0	± 40.5	83.3	± 34.0	0.94
Mannitol Salt					
Staphylococci-Micrococci (STM)	217.4	± 78.8	195.7	± 62.9	0.99
Saboraud Dextrose					
Yeasts (YST)	399.3	±160.1	397.3	±166.6	0.99

Legend: [a]SEM, Standard error of the mean
[b]$P < 0.05$ for difference between means.

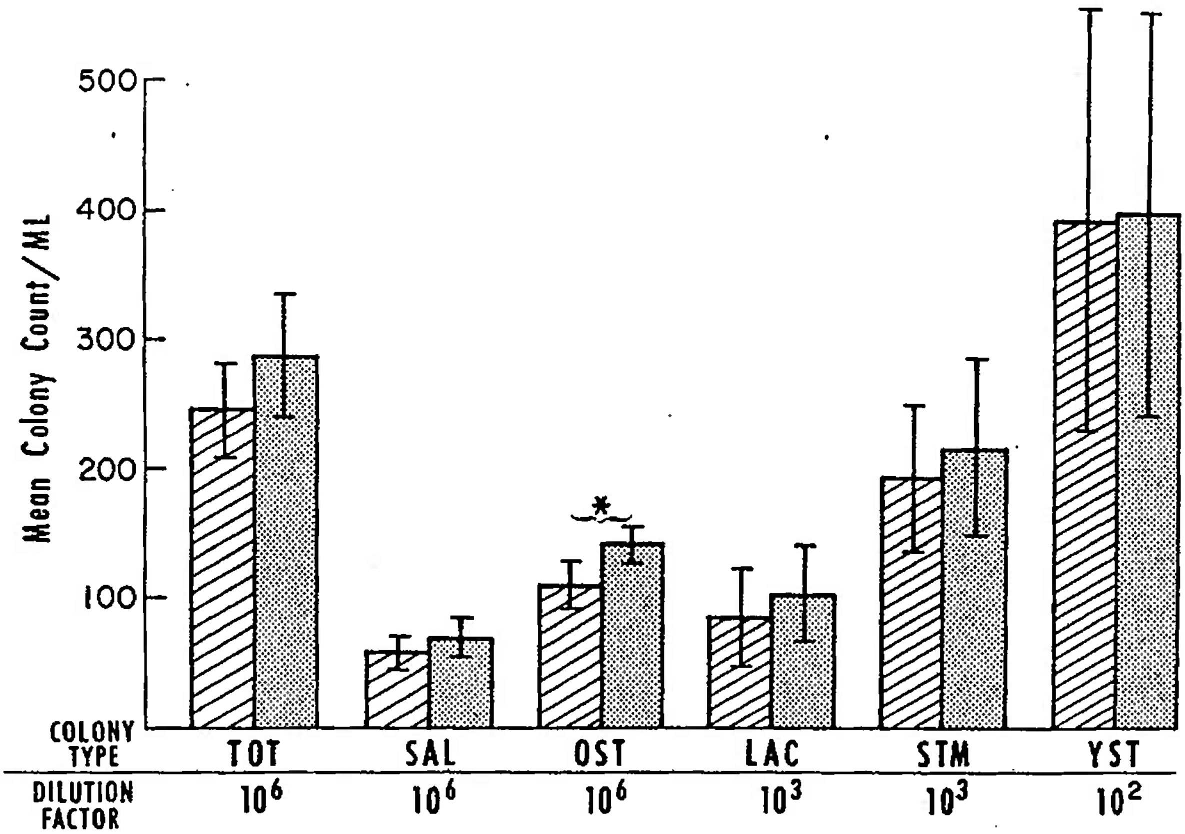

Fig. 1. Mean salivary bacterial counts after dispersion of whole saliva by Vortex and Jay Shaker techniques.

Legend:	*TOT*	–	*Total Colony Count on Brain Heart Infusion Agar.*
	SAL	–	*Differential count of Streptococcus salivarius on Mitis-Salivarius Agar.*
	OST	–	*Other Streptococcal types on Mitis-Salivarius Agar.*
	LAC	–	*Total Lactobacilli Count on Rogosa SL Agar.*
	STM	–	*Total Count on Mannitol Salt Agar (Staphylococci-Micrococci).*
	YST	–	*Total Yeast Count on Sabouraud Dextrose Agar.*

 – *Vortex dispersion technique*

 – *Jay dispersion technique*

 – *±Standard error of the mean*

* – *Probability difference level < 0.05.*

Paired counts from Jay-treated and Vortex-treated samples were highly correlated: Total Colony Count (r=0.82), Lactobacilli (r=0.93), Staphylococci-Micrococci (r=0.99), and Yeasts (r=0.99) (Table I). Paired Streptococcus salivarius counts at 24 hours and OST counts at 5 days produced weaker correlation coefficients of 0.62 and 0.44, respectively.

DISCUSSION

The widespread use of the Jay Shaker for the dispersion of whole saliva apparently rests more on tradition than on its efficiency and ease of use. The purpose of this study was to determine the degree of correlation between bacterial counts following Vortex dispersion and Jay dispersion of paired homologous whole saliva samples. In this regard three important features were demonstrated.

1. Mean plate counts following Vortex dispersion were systematically greater than those obtained using the Jay dispersion method. No statistically significant differences were observed with the exception of OST counts made after 5 days incubation of Mitis-salvarius Agar. This strongly suggests that the Vortex dispersion method more efficiently breaks up clumps and/or uniformly distributes microorganisms contained in the treated saliva samples.

2. The strong correlation between paired plate counts for the Jay and Vortex-treated saliva samples demonstrated a practical and useful correspondence between the two methods.

3. The advantages of the Vortex technique relative to (a) shorter specimen processing time; (b) ease of handling dilution blanks; (c) smaller volumes of diluent; (d) single dilution series per saliva sample as contrasted to the double dilution series required for the Jay technique; and (e) the greater mixing efficiency of dilution blanks reflected in systematically higher plate counts should reduce total sample variance through greater control of experimental error.

No obvious explanations are readily available for the significant difference observed between OST counts obtained by the two dispersion methods. However, some differences in mixing efficiencies between the two methods and hence differences in particle distribution upon subsequent sampling (preparing dilutions) would be expected. Since the OST group most probably represents a relatively heterogenous population, whose individual growth characteristics would be affected by physical as well as nutritional factors, it is not inconceivable that the greater force inherent in Vortex dispersion may have resulted in chain disruption which in turn would be reflected by higher individual counts. These differences were probably accentuated by the long incubation period. Also, Mitis-Salivarius Agar, while traditionally used as a selective medium, will support both maintenance and minimal growth of a variety of bacteria commonly present among the oral flora. Thus, prolonged incubation (5 days) would allow inhibited organisms to contribute to the OST count.

CONCLUSION

Bacterial counts derived from several different media revealed that in general, no significant differences existed between the two dispersion techniques employed. The only notable exceptions were counts on Mitis-Salivarius Agar plates. Strong correlations existed between counts determined by the two methods.

Relative factors of portability, compactness and decreased specimen processing time indicate that the Vortex dispersion technique is to be preferred to the Jay method for purposes of general laboratory and field utilization.

Quantitative similarities in bacterial counts observed in this study suggest the feasibility of relating salivary bacterial data derived using Vortex dispersion to data from studies using Jay dispersion.

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13. ABSTRACT

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Various methods of counteracting this agglutination effect have been investigated, ranging from the treating of saliva with dilute sodium hydroxide or 1% horse serum to a variety of mechanical dispersion techniques.

Although none of the techniques studied have proven completely satisfactory, the method most often employed in quantitative experiments has traditionally been that of mechanical shaking described by Jay in 1927. A newer, and more efficient instrument, the Vortex Genie, is now commonly used in laboratories to disperse and suspend bacterial cultures. Most workers in the field of salivary bacteriology are reluctant to adopt this instrument since comparisons with the traditional technique are not available. Because the Vortex Genie is more economical in time, space and money this study sought to determine correlations between the two methods as a basis for using the Vortex Genie in future salivary bacterial research.

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